A Cleaved Amplified Polymorphic Sequence (CAPS) Marker Associated with Root-Knot Nematode Resistance in Sugarbeet

J. J. Weiland* and M. H. Yu

ABSTRACT

Resistance to root-knot nematode (*Meloidogyne* spp.) previously was introgressed into sugar beet (*Beta vulgaris* L.) from wild beet [*B. vulgaris* ssp. *maritima* (L.) Arcang] and was demonstrated to be dominant and simply inherited. Since resistance conferred by this gene was effective against six different *Meloidogyne* spp. tested, the locus was designated *R6m*-1. An interpollinated progeny population of resistant heterozygotes segregating for *R6m*-1, was exposed to nematodes in a greenhouse and rated for root knot disease symptoms. Resistance vs. susceptibility segregated at approximately a 4:1 ratio and 120 resistant roots and 48 susceptible roots were chosen for the generation of molecular markers linked to the resistance trait. Bulked DNA samples prepared from shoots sprouting from the selected plants were subjected to random amplified polymorphic DNA (RAPD) analysis and yielded a marker of 600 base pair (bp) that was highly associated with resistance. Sequence analysis of the 600-bp product led to the design of DNA primers for specific amplification of a 580-bp product, the generation by polymerase chain reaction (PCR) of which occurred in plants both susceptible and resistance to nematode. Comparison between the sequences generated from resistant plants and susceptible plants revealed numerous nucleotide substitutions. One base substitution associated in repulsion with resistance conditioned the existence of a recognition site for cleavage by the restriction endonuclease *Mse*I. Amplification and cleavage of the product with *Mse*I yielded a CAPS marker designated NEM06 that cosegregated with resistance to the root knot nematode. Computer-assisted translation and comparison with sequences in public databases indicates that the marker DNA sequence encodes a protein with high sequence similarity to a plant transcription factor.

Root-knot nematode causes disease in sugar beet and other crops within the genus *Beta*. Although nematodes are serious pests of crops worldwide, they affect sugar beet production in comparatively localized growing regions. Nonetheless, in areas where *Meloidogyne* spp. occur, they can be a serious problem and in some cases result in crop failure. Of economical significance to sugar beet are the root-knot nematodes *M. incognita* Chitwood, *M. arenaria* Chitwood, *M. javanica* Chitwood, *M. hapla* Chitwood, *M. fallax* Karssen, and *M. chitwoodi* (Whitney and Duffus, 1986). In the USA, *M. incognita* and *M. javanica* cause the greatest sugar beet crop losses, primarily in production regions south of the 40th parallel and in the southwestern USA, including California (Altman and Thomason, 1971; Whitney and Duffus, 1986; Duffus and Ruppel, 1993).

Disease of sugar beet caused by root-knot nematode is manifested by galls that form on lateral roots and the tap root (Whitney and Duffus, 1986). In warm climates with long growing seasons, early colonization of young roots can lead to severe crop losses through plant death. In older beets, nematode infection and galling increase the possibility of secondary invasion by root pathogens and is consequent with leaf chlorosis, further suppressing yield potential. In the absence of genetic resistance in commercial varieties, both crop rotation and nematicides have been used to manage root-knot nematode. However, because of nematodes' wide host range and increasing restrictions on nematicide use, control of *Meloidogyne* spp. in sugar beet fields continues to be problematic.

Breeding for resistance to root knot nematode in sugar beet has been a relatively recent endeavor. Yu (1995) reported the existence of root-knot nematode resistance in rare strains of *B. vulgaris* ssp. *maritima*. Further investigation resulted in the discovery of an isozyme marker associated with a gene for resistance to root-knot nematode in Mi-1 *Beta* (Yu et al., 2001). By contrast, the sugar beet cyst nematode, *Heterodera schachtii* Schm., has been the subject of intense research effort, culminating in the positional cloning of the $Hs1^{pro-1}$ gene for resistance to a limited number of races of this organism (e.g., Cai et al., 1997). The difference in research effort is due to the fact that infestation of sugar beet fields with cyst nematode is more widely spread than root-knot nematode and leads to more serious, recurring losses to growers.

Molecular markers have gained favor in plant biology, including aspects of plant breeding. A molecular marker may serve as a guide in determining which plants should be advanced within a program for varietal improvement (Knapp, 1998). In addition, markers that tag disease resistance genes can be used to map the locations of genes in genomes, assess the extent of clustering of such genes on chromosomes, and aid in the cloning of genes by map-based strategies (Hammond-Kosack and Jones, 1997). By combining the availability of DNA-based markers with annotated genome sequence data, the localization of markers on genome maps promises to be greatly accelerated.

In this report, we sought to obtain molecular markers linked to nematode resistance present in the M66 Series of sugar beet described by Yu (1996). A 0.6-kbp RAPD marker tightly linked with resistance to root-knot nematode was obtained from the study. The characterization of this marker and its conversion to a CAPS (Konieczny and Ausubel, 1993) marker (NEM06) capable of distin-

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guishing plants homozygous and heterozygous for the *R6m*-1 gene is described. It is anticipated that the NEM06 marker will be useful in plant breeding and genome characterization in sugar beet.

MATERIALS AND METHODS

Plant Germplasm and Families

Sugar beet population 1568 used in this study was developed from hybridization between *Meloidogyne* spp.-resistant germplasm M66, (PI 586688; Yu, 1996; Yu et al., 1999) and susceptible sugar beet line C37 (Lewellen et al., 1985). The F_1 plants with nematode resistance were selected and backcrossed to C37 for five consecutive generations. At this point, all resistant plants were heterozygous for resistance to root-knot nematode. Plant materials used in the molecular marker analysis were produced by interpollinating 20 or more resistant heterozygous plants in isolation chambers. Progeny of the interpollinated populations included homozygous and heterozygous resistant plants and susceptible homozygous individuals.

Inoculation Procedures and Resistance Analysis

Sugar beet seedlings were germinated in 3- × 17-cm polyethylene containers holding 110 cm^3 of 1:9 (v/v) soil and sand mixture in the greenhouse. One multigerm sugar beet seed was planted per container and only one seedling that emerged was saved. Seedlings were inoculated at four- to six-leaf stage with 800 newly hatched second-stage juveniles (J2) per plant in a 1 mL suspension. Seven days later, an additional 400 J2 juveniles were applied. The inoculated plants were rearranged weekly, maintained at 24 to 28°C above heat pads, and fertilized weekly with approximately 40 mL/plant of an aqueous solution of 20-20-20 (N-P-K) diluted 250 times. Roots were washed and examined for root gall and protuberance formations at around 40 d after the final inoculation. Gall and protuberance counts were classified into 0 (zero), 1 to 4 and 5 to 10 where galls typically were less than 2 mm in diameter, 11 to 30, 31 to 100, and >101 groups. Individual plants with 10 or fewer galls were rated as resistant and those with 11 or greater galls were considered susceptible. The rate of nematode reproduction is positively associated with the number of root galls formed and the criterion for nematode resistance has also proved practical in our breeding research (Yu, 1995).

RAPD Analysis and Marker Refinement

Sugar beet roots individually classified as resistant or susceptible to nematode were shipped to the USDA-Sugar beet Pathology Lab at Fargo, ND, where they were potted and allowed to resprout. Young shoots were harvested from the plants and DNA was prepared from them according to the method of Doyle and Doyle (1987). After quantitation of the DNA by spectrophotometry, the samples were adjusted to a concentration of 10 ng/μL. Pooled samples for bulk segregant analysis (BSA; Michelmore et al., 1991) consisted of DNA from seven individuals. Three pools each were made from 21 resistant and 21 susceptible plants, respectively, and these were subjected to the PCR-based RAPD (Williams et al., 1990). Reactions consisted of 40 ng pooled genomic DNA, 0.2 μ*M* of each arbitrary decamer primer (Operon Technologies, Inc., Alameda, CA), 10 m*M* TRIS-HCl (pH 9.0), 50 m*M* KCl, 0.1% (v/v)Triton X-100, 2.0 m*M* $MgCl_2$, 0.1 m*M* each of d(G,A,T,C)TP and 2 units of Taq DNA polymerase (Promega, Madison, WI). Amplified DNA was separated on 1.6% (w/v) agarose gels in Tris-borate-EDTA buffer (Sambrook et al., 1989), stained with ethidium bromide, and photographed with a ChemImager 4000 gel documentation system (Alpha-Innotech Inc., San Leandro, CA).

Amplified DNA of the candidate RAPD marker for NEM06 was gel-purifed by standard techniques and cloned into the commercial plasmid vector pCR2.1 (Invitrogen, Calsbad, CA). Minipreparations of DNA containing the insert were sequenced by the Iowa State University DNA sequence facility. Alignment of the DNA sequences from multiple clones was made by means of the Clustal W (Thompson et al., 1994) and translation of the sequence was performed by DNAaid software. A BLAST search on the protein sequence encoded by the marker DNA was done by standard tools within the NCBI Sequence analysis site (Altschul et al., 1990) with further protein sequence alignments done by Clustal W (Thompson et al., 1994).

Oligonucleotide primers (Nem06FWD: 5′-TGCCGAGCT GCTTGACGGGTTGTC-3′) and Nem06REV: 5′-GTTTCG CTCCTCAGAATTGCTGAAG) designed on the basis of the sequence of the cloned 0.6-kbp RAPD product were synthesized by Life Technologies, Inc.(Gaithersburg, MD) and used to amplify a truncated length version of the 0.6-kbp product. The PCR reactions were composed of 40 ng of genomic DNA, 50 ng of each Nem06FWD and Nem06REV primers, 10 m*M* TRIS-HCl (pH 9.0), 50 m*M* KCl, 0.1% (v/v) Triton X-100, 1.5 m*M* $MgCl_2$, 0.1 m*M* each of d(G,A,T,C)TP, and 2 units of Taq DNA polymerase (Promega Corp., Madison, WI). Amplified DNA was electrophoresed in a 1.0% (w/v) agarose gel in Tris-borate-EDTA buffer, stained with ethidium bromide, and photographed.

A DNA sequence polymorphism specifying recognition by the restriction endonuclease *Mse*I was detected by digestion of the NEM06 amplified DNA product. After performing PCR with primers Nem06FWD and Nem06REV, 10 μL of amplified DNA were mixed with 10 μL of a mixture containing 1 unit of *Mse*I and buffer supplied by the manufacturer and the contents were incubated at 37°C for 2 h. Digested DNA was fractionated on a 5% (w/v) polyacrylamide gel containing Tris-borate-EDTA buffer, stained with ethidium bromide, and photographed.

RESULTS AND DISCUSSION

Sugar beet population 1568 was the fifth backcrossed generation progeny of the *Meloidogyne* spp.-resistant germplasm M66. Segregation of root-knot nematode resistance and susceptibility in this backcrossed population was about equal, 66 vs. 68 plants, suggesting that resistance is inherited as a single dominant gene (χ^2 = 0.030, P = 0.863; Table 1). On the other hand, when those resistant individuals were interpollinated in groups, the resultant progeny plants segregated for resistance and susceptibility at approximately 4:1 ratio (χ^2 = 0.067, P = 0.797; Table 1). All of the susceptible control plants were noticeably galled with counts of 11 or greater galls per plant. The experiment was repeated two additional times with similar results. Deviation from the expected 3:1 (resistant:susceptible) ratio may have resulted from the classification of some susceptible plants as resistant due to inadequate galling in tests of the intercross progeny or may reflect segregation distortion not manifest in the backcross tests. In either case, the results favor a model wherein resistance to

Table 1. Segregation of resistance in backcross and intercross sugarbeet populations after inoculation with root-knot nematode.

Type of pollination	No. plants tested	Galls and protuberances per plant						Res.† total	Sus.† total	χ^2	*P*-value
		0	1-4	5-10	11-30	31-100	>101				
Back crossing	134	21	43	2	1	14	53	66	68	0.030‡	0.863
Intercrossing	374	46	159	96	25	21	27	301	73	0.067‡	0.797
Checks	65				1	18	46				

† Plants with 0 or fewer than 10 galls and protuberances/plant were classified as resistant; those with 11 or more galls were susceptible.
‡ Calculated on a predicted 1:1 (resistant:susceptible) ratio for back-cross segregation and a 4:1 ratio for inter-cross segregation.

root-knot nematode in M66 *Beta* is likely dominant and simply inherited. Because the locus confers resistance in sugar beet to 6 species of nematode in the genus *Meloidogyne*, it is given the name *R6m*-1.

Several RAPD markers associated both in coupling and repulsion with resistance to root-knot nematode were observed in the study. Amplification of a 0.6-kb DNA fragment with a combination of the primers A02 and B01 was particularly robust in the RAPD profiles generated from the population and this was chosen for conversion to either a sequence tagged site (STS) or CAPS marker (Fig. 1). Sequence analysis of the cloned 0.6-kb marker and comparison with entries in the Genbank database (version verified 22 May 2002) revealed the possibility that it spans a part of a plant transcription factor gene (Fig. 2). Whether these sequences are part of a functional gene or have any direct or indirect role in nematode resistance remains to be determined. The sequence of this amplified product has been submitted to the Genbank Database with accession AY210437.

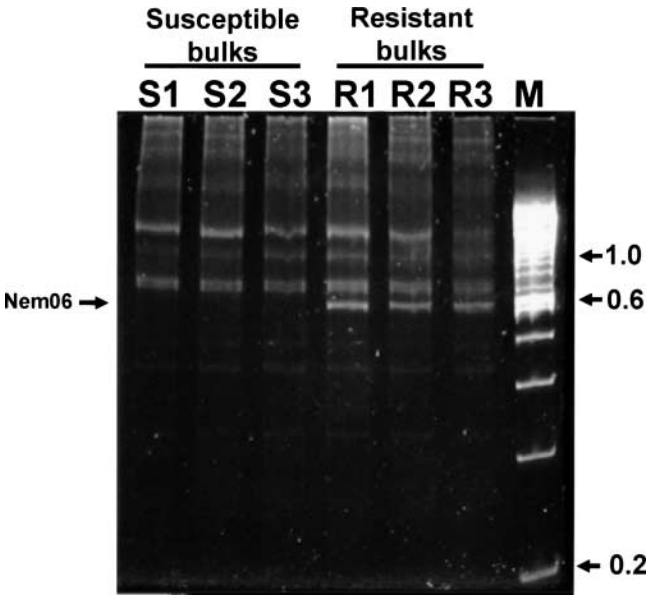

Fig. 1. Generation of a DNA marker associated with sugar beet resistance to root-knot nematode. Bulked DNA samples prepared from susceptible (S1, S2, S3) and resistant (R1, R2, R3) sugar beet plants were subjected to RAPD analysis using the decamer primer pair OPA02 and OPB01. Amplified DNA products were separated on a 1.6% (w/v) agarose gel, stained with EtBr and photographed. The approximately 0.6-kb band seen only in the lanes representing the resistant bulk samples and indicated by the arrow (NEM06) was cloned for sequence analysis and subsequent conversion to a CAPS marker. The 100-bp ladder was included as a size standard (lane M) with the sizes shown listed in kilobasepairs.

To generate a single amplified product that represented the sequence, DNA primers designated Nem06-FWD and Nem06REV were designed on the basis of the nucleotide sequence obtained (Fig. 2). The Nem06FWD and Nem06REV primers were able to amplify the corresponding 0.6 kb DNA product from the genome of sugar beet DNA (not shown). However, the amplicon produced by these primers was generated in both nematode-susceptible and nematode-resistant samples. This suggests that the polymorphism detected by RAPD analysis was not due to unique DNA sequences associated with nematode resistance, but that minor nucleotide substitutions exist within the marker sequence between the resistant and susceptible alleles of this gene. Sequence analysis then was performed on DNA amplified from susceptible and resistant plants with Nem06-FWD and Nem06REV and alignments were made between NEM06 marker sequences generated from these two phenotype classes (Fig. 2).

Several nucleotide substitutions exist between the marker sequences of the two classes with one substitution at nucleotide 208 conditioning the presence (susceptible) or absence (resistant) of an *Mse*I restriction endonuclease site (Fig. 2). Presence of this site in the NEM06 amplicon derived from nematode-susceptible sugar beet was validated by comparing the fragmentation pattern after digestion with *Mse*I of DNA amplified from pooled DNA of susceptible and resistant sugar beet with Nem06FWD and Nem06REV (not shown). Nematode resistant plants in sugar beet population 1568 are a mixture of those homozygous and heterozygous for the nematode resistance gene. Thus, application of the Nem06 CAPS marker to individuals of the population should yield some plants that produce both *Mse*I noncleavable and cleavable PCR products (heterozygotes) as well as those that produce only the noncleavable product (homozygotes). This can be seen in Fig. 3, where either a mixture of the two digestion products or a single product is seen in lanes representing individual plants. The association between the CAPS marker and the resistance phenotype is summarized in Table 2. No recombination between the gene conferring resistance to nematode and the Nem06 marker was detected in the progeny used. However, a larger sample size would give a better indication of the ability for the Nem06

A

TGCCGAGCTGCTTGACGGGTTGTCAATATGCGGTGCATTTGTTGCAAAGTCTGCTGCCTCAAAAGATCAGCCT
AACAATATTTAAAACAAAAGAAAGGGAACTCAAATG***TTAG***ATAATTGTAGGTCAGGAAATGGAACAAGTTTCA
AGATTTTTTACATAAGAAACCATACCTGGTGAACAAAGTTCTCTAGTGTGGCAAGCTTGCCCATAGCAATCGC
CATTTGACCCATGTAATCAGCAACAACTCCAGTATTAGATGGACTGAGACAGTTTGCAGAAAGTGTATCAACA
AGGGATTGTTGCAGCGCTTCCATTCCCTGTGATAAAGCATCCTCGGCTTGTTGAGAGGATTGCTGCAAATTGC
ATATTTCCCCATCAATTGCTGCTCTGTCAGAAGGTTCAAGGTGGTTCACTAGTATCTGATATTCCAACAACAA
AAAACATAATTTTTTAAAAAAATTAAAAGCGCTTCAATTCAGTCCTAAAGTAGAGGTGTTTGGGCAAGAAAAA
AGAGACAAGATTGTGTCTGGGTGCTTAAAAGTAGTAATGAC**CTTCAGCAATTCTGAGGAGCGAAAC**

B

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CLUSTAL W (1.82) multiple sequence alignment

A.t. KILGNHVDPLTDQQLIGICNLQQSSQQAEDALSQGMEALQQSLLETLSSASMGPNSSANV

L.e. KLLVNQLEPLTEQQLAGIYNLQQSSHQAEDALSQGMEALQQSLAETLANGSPATEGSSGV

B.v. QVLVNHLEPLTEQQLMGICNLQQSSQQAEDALSQGMEALQQSLVDTLSANCLSPSNTGVV
     ::* *:::***:*** ** ******:****************** :**: . ....:.  *

A.t. ADYMGHMAMAMGKLGTLENFLRQ

L.e. ANYMGQMAMAMGKLGTLEGFLRQ

B.v. ADYMGQMAIAMGKLATLENFVHQ
     *:***:**:*****.***.*::*
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C

Alignment of Resistant and Susceptible DNA fragments

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Nematode Susceptible #2
179  AAACAAAAGAAAGGGAACTCAAATGTTAAATAATTGTAGGTCAGGAAATGGAACAAGTTT    239
Nematode Susceptible #3
179  AAACAAAAGAAAGGGAACTCAAATGTTAAATAATTGTAGGTCAGGAAATGGAACAAGTTT    239
Nematode Susceptible #1
179  AAACAAAAGAAAGGGAACTCAAATGTTAAATAATTGTAGGTCAGGAAATGGAACAAGTTT    239
Nematode Resistant #5
179  AAACAAAAGAAAGGGAACTCAAATGTTAGATAATTGTAAGTCAGGAAATGGAACAAGTTT    239
Nematode Resistant #6
179  AAACAAAAGAAAGGGAACTCAAATGTTAGATAATTGTAGGTCAGGAAATGGAACAAGTTT    239
Nematode Resistant #7
179  AAACAAAAGAAAGGGAACTCAAATGTTAGATAATTGTAGGTCAGGAAATGGAACAAGTTT    239
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Fig. 2. Sequence analysis of the 0.6-kb amplified DNA marker associated with resistance to root knot nematode. In A, the complete nucleotide sequence is shown with the decamer primer binding sites (underlined) and the subsequent CAPS marker primer binding sites (block bold text) indicated within. The four nucleotide sequence (bold italics) comprising an *Mse*I resistriction site polymorphism difference between alleles of the marker is also shown. Translation of a portion of the DNA sequence into encoded protein sequence (B) indicating similarity to the proteins encoded by the *PERIANTHA* gene from *Arabidopsis thaliana* (A.t.) and the NIP1 gene from *Lycopersicum esculentum* (L.e.), both putative transcription factors. An alignment (C) of a portion of the NEM06 marker sequence amplified by the Nem06fwd and Nem06rev primers from both susceptible and resistant sugar beet. The four-base recognition sequence for the restriction enzyme *Mse*I is shown in bold with boxes surrounding the nucleotide difference between the alleles that determines the inability ("G") or ability ("A") for this enzyme to cleave the DNA.

Table 2. Association between marker NEM06 and resistance to Meloidogyne spp. in sugarbeet.

Plant Class†	Total number‡	NEM06 (coupling)§	NEM06 (repulsion)¶	Percent association
Nem. Suscept.	21	0	21	100
Nem. Resist.	75	75	0	100

† Plants were rated as either highly resistant or highly susceptible and represent a portion of the population screened for phenotype.
‡ The total number of roots in each class analyzed for NEM06 association.
§ All resistant plants had at least one copy of the marker allele that is non-cleavable by *Mse*I.
¶ All susceptible plants were homozygous for the marker allele that is cleavable by *Mse*I.

CAPS marker to predict the presence of nematode resistance in sugar beet populations.

The CAPS marker described here may offer a rapid means of identifying seedlings that should be maintained in a nematode resistance breeding program. Furthermore, the marker should prove useful in localizing the resistance gene in the genome of *Beta*. The availability of this marker, along with the isozyme marker previously described for a second resistance gene or allele to root-knot nematode in Mi-1 *Beta* (Yu et al., 2001), provide new tools for marker-assisted selection and improvement of sugar beet.

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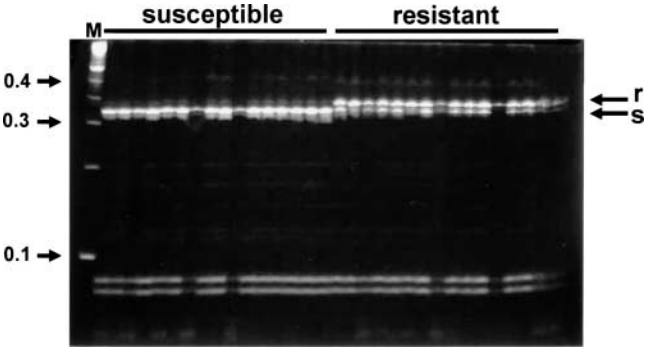

Fig. 3. Marker status of sugar beet plants rated for resistance or susceptibility to root-knot nematode. Amplified Nem06 marker DNA from 16 sugar beet plants either susceptible or resistant to root-knot nematode was digested with *Mse*I and the products were separated by electrophoresis on a 5% (w/v) polyacrylamide gel. Arrows denote the migration of the marker allele (r) that is coupled with resistance and the marker allele (s) associated in repulsion with resistance. The 100-bp ladder was included as a size standard (lane M) with the sizes shown listed in kilobasepairs.

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